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(54) Title: METHOD FOR ANALYZING OLIGONUCLEOTIDE ANALOGS

(57) Abstract

Disclosed is a substrate useful for separating unmodified and modified mononucleotides and oligonucleotides. The substrate includes at least 12 % (weight: volume) polymer in at least 5 M urea and at least 32 % (volume: volume) organic solvent, the organic solvent being a chemically stable liquid at room temperature and having a dielectric constant of at least 20. Also provided is a method of separating unmodified and modified mononucleotides and/or oligonucleotides utilizing this substrate.

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METHOD FOR ANALYZING OLIGONUCLEOTIDE ANALOGS

CROSS-REFERENCE TO RELATED APPLICATION

This patent application is a continuation-in-part of patent application Serial No. 08/032,856, filed March 16, 1993, entitled ANALYTICAL TECHNIQUE FOR OLIGONUCLEOTIDE ANALOGS, which is a continuation-in-part of patent application Serial No. 07/991,466 of the same title, filed December 16, 1992.

10

FIELD OF THE INVENTION

This invention relates to methods for separating mononucleotides and oligonucleotides. More particularly, this invention relates to the separation and characterization of modified and unmodified mononucleotides and oligonucleotides by high performance capillary electrophoresis.

15

BACKGROUND OF THE INVENTION

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Oligonucleotides that are complementary or "antisense" to specific genes or RNA sequences are relatively small, synthetic molecules having an average molecular weight of about 10 kilodaltons (kD). These antisense molecules have had widespread use in the field of selective gene regulation with consequent therapeutic implications. Phosphate backbone modification of such oligonucleotides provides nuclease resistance and greatly enhances the usefulness of these

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analogs. Such modifications include the substitution of phosphodiester internucleotide linkages with linkages such as methylphosphonates (Murakami et al. (1986) *Biochem.* 24:4041-4046; 5 Agrawal et al. (1987) *Tetrahedron Lett.* 28:3539-3542; Sarin et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7448-7451), phosphorothioates (Burgers et al. *Biochemistry* 18:592-596; Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083; Agrawal et al. 10 (1989) *Nucleosides and Nucleotides* 8:819-823; Agrawal et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:7790-7794), and phosphoramidates (Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083; Agrawal et al. (1989) *Nucleosides and Nucleotides* 8:819-823).

15 Of special interest are phosphorothioate analogs in which one non-bridging oxygen atom has been substituted for a sulfur atom on the phosphate group in one or more internucleotide phosphodiester linkages. This modification is a conservative substitution which increases nuclease resistance without significantly impairing the hybridization of the antisense molecule with target mRNA. As synthesized, these modified 20 oligonucleotides or analogs are usually found as diastereomeric mixtures due to chirality at their phosphorous group. In a context of new drug research, development and manufacturing of such 25 analogs requires that the issues of oligomer length, base composition, base sequence, chemical purity, and stereochemical purity be successfully 30 addressed.

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Synthetic oligonucleotides are presently used in most laboratories using molecular biology techniques. As synthesized, these oligonucleotides generally exist as mixtures of truncated oligonucleotides in addition to the desired oligonucleotide. Since the purity and chemical identity of a particular oligonucleotide is crucial to many applications, the ability to characterize and separate synthetic oligonucleotides analogs on a routine basis is important.

The absolute length and the degree of length heterogeneity of prepared oligonucleotides have been assessed by electrophoresis in high resolution denaturing polyacrylamide slab gels (PAGE) (see, e.g., *Current Protocols in Molecular Biology*, Green Publishing and Wiley Interscience, N.Y., 1988) and by capillary gel electrophoresis through cross-linked polyacrylamide (6% T, 5% C) gels (Hjerten (1967) *Chromatogr. Rev.* 9:122-213) containing from 10% to less than 30% (vol.:vol.) formamide (Rocheleau et al. (1992) *Electrophoresis* 13:484-486). Detection of oligonucleotides separated on such gels has been accomplished by autoradiography and laser-induced fluorescence. These methods have not proven suitable for separating modified oligonucleotides. Furthermore, some of these gels, once used, are not easily removable from the capillary. To remedy this problem, gels containing up to 5% acrylamide monomer have been polymerized before filling the capillary (EPO 497 480). Ultrathin

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analogs. Such modifications include the substitution of phosphodiester internucleotide linkages with linkages such as methylphosphonates (Murakami et al. (1986) *Biochem.* 24:4041-4046; 5 Agrawal et al. (1987) *Tetrahedron Lett.* 28:3539-3542; Sarin et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7448-7451), phosphorothioates (Burgers et al. *Biochemistry* 18:592-596; Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083; Agrawal et al. 10 (1989) *Nucleosides and Nucleotides* 8:819-823; Agrawal et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:7790-7794), and phosphoramidates (Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083; Agrawal et al. (1989) *Nucleosides and Nucleotides* 8:819-823).

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Synthetic oligonucleotides are presently used in most laboratories using molecular biology techniques. As synthesized, these oligonucleotides generally exist as mixtures of truncated oligonucleotides in addition to the desired oligonucleotide. Since the purity and chemical identity of a particular oligonucleotide is crucial to many applications, the ability to characterize and separate synthetic oligonucleotides analogs on a routine basis is important.

The absolute length and the degree of length heterogeneity of prepared oligonucleotides have been assessed by electrophoresis in high resolution denaturing polyacrylamide slab gels (PAGE) (see, e.g., *Current Protocols in Molecular Biology*, Green Publishing and Wiley Interscience, N.Y., 1988) and by capillary gel electrophoresis through cross-linked polyacrylamide (6% T, 5% C) gels (Hjerten (1967) *Chromatogr. Rev.* 9:122-213) containing from 10% to less than 30% (vol.:vol.) formamide (Rocheleau et al. (1992) *Electrophoresis* 13:484-486). Detection of oligonucleotides separated on such gels has been accomplished by autoradiography and laser-induced fluorescence. These methods have not proven suitable for separating modified oligonucleotides. Furthermore, some of these gels, once used, are not easily removable from the capillary. To remedy this problem, gels containing up to 5% acrylamide monomer have been polymerized before filling the capillary (EPO 497 480). Ultrathin

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slab gels (less than 100  $\mu\text{m}$  in thickness) have also been used for high speed DNA sequencing (Brumley et al. (1991) *Nucleic Acids Res.* 19:4121-4126; Ansorge et al. (1990) *Nucleic Acids Res.* 18:3419- 5 5420). Alternative separation methods include ion exchange chromatography, reversed phase high pressure liquid chromatography (HPLC), and gel high performance capillary electrophoresis (see, e.g., Edge et al. (1981) *Nature* 292:756-762; U.S. 10 Patent No. 4,865,707).

Oligonucleotides with phosphorothioate linkages are more difficult to resolve than phosphodiester-linked DNA due to the existence of diastereomer isomers ( $2^n$ , where n = the number of chiral centers, which is equivalent to the number of phosphate groups). In addition, difficulty in resolution may be due to increased hydrophobicity of the former. These molecules, when separated, 15 interact hydrophobically with ion exchange column supports and in many cases co-elute. Thus, they 20 cannot be separated by the above methods in their existing formats.

The separation of phosphorothioate oligonucleotide analogs is problematic for other reasons as well. When phosphorothioate oligonucleotides are assembled using either methoxyphosphoramidite or H-phosphate chemistry, 25 they are in the form of diastereomeric mixtures due to chirality at their phosphorous groups. As a result, although they migrate through polyacrylamide gels and HPLC columns like their 30

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corresponding phosphodiester counterparts,  
phosphorothioate oligonucleotides give broader  
peaks and run more slowly than phosphodiesters  
because of their increased hydrophobicity or  
secondary structure. They are also known to  
interact with the HPLC column support. In  
addition, phosphorothioates run into  
stereochemical problems when separated by reversed  
phase HPLC. General analytical methods have not  
been devised for establishing the ratio of the  
optical isomers at each unsymmetrical substitution  
phosphorous linkage in an analog having many such  
sites of local chirality.

HPLC of oligodeoxyribonucleotides containing  
one or two phosphorothioate internucleotide  
linkages using a reversed-phase column (RP-HPLC)  
has been reported (Stec et al. (1985) *J. Chromatogr.*  
326:263-280; Agrawal et al. (1990) *Nucleic Acids Res.*  
18:5419-5423). However, this method is of limited  
use because of the small differences in the  
hydrophobicity of these analogs with increasing  
chain length (Agrawal et al. (1990) *J. Chromatogr.*  
509:396-399).

Separation of oligodeoxyribonucleotide  
phosphorothioates containing 20 or fewer  
nucleotides has also been achieved by HPLC on  
strong and weak anion-exchange (SAX and WAX)  
columns (Cohen et al. (1993) *J. Chromatogr.* 638:293-  
301). Unfortunately, oligonucleotide  
phosphorothioates containing more than 20  
nucleotides can not be analyzed by this method

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because of their strong interaction with the SAX or WAX medium. Thus the separation of oligonucleotide phosphorothioates by this method is limited by its oligonucleotide length dependency.

Length-dependent separation of phosphorothioate analogs by HPLC using a WAX column has also been accomplished by Meletev et al. (*Analyt. Biochem.* (1993) 200:342-346). However, the peaks obtained were broader than those obtained for their phosphodiester counterparts, possibly because of their diastereomeric backbone. Ion-pair HPLC has also been used to analyze oligonucleotide phosphorothioates (Bigelow et al. (1990) *J. Chromatogr.* 533:133-140), but length-dependent separation was not achieved.

Thus, what is needed are better analytical methods of, and substrates for, separating unmodified and modified mononucleotides and oligonucleotides cleanly, rapidly, efficiently, and which are not limited by the size range or modification of the molecules being analyzed. Also, what is needed are better methods of, and substrates for, separating and characterizing oligonucleotides such as those which are partially or totally oxidized.

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SUMMARY OF THE INVENTION

A novel substrate and method of its use have been developed for the separation and characterization of unmodified and modified mononucleotides and oligonucleotides differing by as little as a single base or oxidation. Furthermore, this method enables the separation and characterization of oxidized oligonucleotides and/or oxidized mononucleotides from unoxidized or partially oxidized oligonucleotides and/or non-oxidized mononucleotides. An advantage to the use of this substrate and method is the relative ease by which samples of less than one nanogram per 5 microliter or lower volumes can be conveniently handled with on-line UV detection. Relative to slab gel electrophoresis and conventional gel high performance capillary electrophoresis/on-line UV operation, this new formulation can be very useful 10 for in process analysis as well as for purity assessment of antisense nucleotides in the pharmaceutical industry.

15

As used herein, an "oxidized oligonucleotide" 20 is one having phosphodiester internucleotide linkages. A "partially oxidized oligonucleotide" 25 is one which has the oxygen in the nonbridging position substituted for another atom or chemical group at some but not all of its internucleotide linkages. An oligonucleotide which is 30 "unoxidized" is one in which the nonbridging oxygen in every phosphate group is substituted with another atom or chemical group.

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The substrate of the invention includes at least 12% (weight:volume) polymer, which, in preferred embodiments is no more than 1% cross-linked, in at least 5 M urea and at least 32% (volume:volume) organic solvent. The organic solvent is a chemically stable liquid at room temperature (from about 19 - 25°C) and has a dielectric constant of at least 20. In one aspect of the invention, the substrate includes from 0 to 10 16.2% water.

Preferable substrate polymers are polyacrylamide, methyl cellulose and derivatives thereof, and polyvinyl alcohol. In one aspect, 15 the substrate of the invention is acrylamide, and in particular, includes at least 12% T polymerized acrylamide (or polyacrylamide). The term "T" refers to the percent of monomers (mass:volume). In one embodiment, the invention includes a 20 substrate containing 18% T polyacrylamide. In another aspect of the invention, the substrate includes polyacrylamide such as 12% to 20% T polyacrylamide, with from about 13% to 18% T being optimal. In another aspect, the substrate 25 contains linear polyacrylamide. The polymer is non-cross-linked in some aspects of the invention, and in others, contains up to 1% cross-linking.

30 Preferable organic solvents making up the substrate and having a dielectric constant of at least 20 are methanol, formamide, acetaldehyde, dimethylsulfoxide (DMSO), ethanol, glycol, acetone, 1-propanol, 2-propanol, 1, 2-propanediol,

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1, 3-propanediol, and glycerol. In two representative embodiments of the invention, the substrate includes either formamide present at a concentration of about 32% to 74% (volume:volume) or DMSO at a concentration of about 32% to 56% (volume:volume). In yet another embodiment, the substrate includes 14% to 56% (volume:volume) DMSO.

10       The invention also provides a method of separating unmodified and modified mononucleotides and oligonucleotides using the above-described substrate. This method includes placing the substrate in a high performance capillary, and  
15      then contacting the substrate with the mononucleotide and/or oligonucleotides to be separated. An electric field greater than 200 volts/centimeter is applied across the substrate in the capillary, and the separated  
20      mononucleotides and/or oligonucleotides are detected. In preferred embodiments of the invention, an electric field of about 400 to 800 volts/cm is applied across the substrate.

25       Molecules capable of being separated by this method include unmodified mononucleotides, unmodified oligonucleotides, mononucleotide analogs, and oligonucleotide analogs, all having from about 1 to 150 bases.

30       As used herein, a "mononucleotide analog" or "modified mononucleotide" is a base, including purines and pyrimidines, or modifications thereof,

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attached to the 1' end of the deoxyribose or  
ribose sugar, or modifications thereof, which is  
attached at its 5' position to a phosphate group.  
Also included as a mononucleotide analog are  
5 cyclic mononucleotides.

The term "mononucleotide analog" or "modified  
mononucleotide" is also meant to encompass 5'-  
10 substituted mononucleotide analogs which include a  
deoxyribose or ribose sugar attached at its 5'  
position to a chemical group other than the  
phosphate group found in native nucleotides.  
Preferable chemical groups include alkyl  
15 phosphonates, phosphorothioates,  
phosphorodithioates, alkyl phosphorothioates,  
phosphoramidates, phosphate esters, carbonates,  
phosphate diesters, carbamates, and phosphate  
triesters. "Mononucleotide analogs" or "modified  
20 mononucleotides" also include "3'-substituted  
mononucleotide analogs" having a deoxyribose or  
ribose sugar attached at their 3' position to a  
chemical group other than the hydrogen found in  
native nucleotides. Also included in the terms  
25 "modified mononucleotide" and "mononucleotide  
analog" are 3',5'-substituted mononucleotides  
having a sugar which, at both its 3' and 5'  
positions is attached to a chemical group other  
than a hydroxyl group (at its 3' position) and  
other than a phosphate group (at its 5' position).  
30 A modified mononucleotide or mononucleotide analog  
may also be unoxidized, i.e., having a  
substitution in one nonbridging oxygen as in a  
phosphorothioate, for example.

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The term "oligonucleotide" includes polymers of one or more ribonucleotide and/or deoxyribonucleotide monomers connected together or linked by at least one 5' to 3' internucleotide linkage.

The terms "modified oligonucleotide" and "oligonucleotide analog," as used herein, encompass a molecule of ribonucleotides or deoxyribonucleotides which are covalently linked via at least one synthetic linkage. A "synthetic internucleotide linkage" is a linkage other than a phosphodiester between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' internucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, carbonates, phosphate triesters, acetamide, and carboxymethyl esters.

The terms "modified oligonucleotide" and "oligonucleotide analog" also encompass oligonucleotides with a modified base and/or sugar. For example, a 3', 5'-substituted oligonucleotide is a modified oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). A modified oligonucleotide may also be a capped

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species. Also encompassed by these terms are unoxidized oligonucleotides or oligomers having a substitution in one nonbridging oxygen per nucleotide in the molecule.

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Synthetic oligonucleotides are also oligonucleotide analogs. A "synthetic oligonucleotide" encompasses polymers of 3' to 5'-linked ribonucleosides, 2'-modified ribonucleosides and/or deoxyribonucleosides having only as many nucleosides as are conveniently chemically synthesized (i.e., up to about 80 - 90). Also encompassed are those oligonucleotides having base or sugar modifications as well as those having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s), multiple ribonucleosides and/or deoxyribonucleosides linked via an internucleotide linkage not found in native DNA, i.e., linkages other than phosphodiester bonds, or having modified bases and/or sugars in various other structural modifications not found *in vivo* without human intervention.

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BRIEF DESCRIPTION OF THE DRAWINGS

5       The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

10       FIG. 1A is an electropherogram demonstrating the separation by HPCE of phosphorothioate failure sequences ranging in length from 1 to 50 bases in length;

15       FIG. 1B is an electropherogram demonstrating the electrophoretic separation by HPCE of phosphorothioate failure sequences ranging in length from 1 to 75 bases in length;

20       FIG. 2 is a calibration plot of migration time of the analogs separated in FIG. 1 versus oligomer length in the analog;

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FIG. 3 is an electropherogram demonstrating the electrophoretic separation of a mixture of 25mer analog (SEQ ID NO:1), 24mer analog (SEQ ID NO:2), and failure sequences resulting from the 5 syntheses of these oligonucleotide analogs. Peak A represents a putative 23mer failure sequence from 24mer (SEQ ID NO:2) synthesis; peak B represents the 24mer analog (SEQ ID NO:2); peak C represents the putative 24mer failure sequence 10 from 25mer analog (SEQ ID NO:1) synthesis; peak D represents a 25mer analog (SEQ ID NO:1); and peak E is unknown;.

FIG. 4A is an electropherogram demonstrating 15 the electrophoretic separation of unoxidized heteropolymers differing in length by one base, using a substrate composed of 12.6% T acrylamide, 7.4 M urea, and 40.5% (volume:volume) DMSO by HPCE (800 V/cm, 3  $\mu$ A) on 10 cm capillaries;

FIG. 4B is an electropherogram demonstrating 20 the electrophoretic separation of oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 25 12.6% T acrylamide, 7.4 M urea, and 40.5% (volume:volume) DMSO by HPCE (800 V/cm, 3  $\mu$ A) on 10 cm capillaries;

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- FIG. 4C is an electropherogram demonstrating the electrophoretic separation of unoxidized oligonucleotide analogs differing in length by only one base, and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 12.6% T acrylamide, 7.4 M urea, and 40.5% (volume:volume) DMSO by HPCE (800 V/cm, 3  $\mu$ A) on 10 cm capillaries;
- FIG. 5A is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base, using a substrate composed of 11.4% T acrylamide, 5.7 M urea, and 48.9% (volume:volume) formamide by HPCE (400 V/cm, 4  $\mu$ A) on 10 cm capillaries;
- FIG. 5B is an electropherogram demonstrating the electrophoretic separation of homopolymers of oxidized polyadenylic acid differing in length by one base, using a substrate composed of 11.4% T acrylamide, 5.7 M urea, and 48.9% (volume:volume) formamide by HPCE (400 V/cm, 4  $\mu$ A) on 10 cm capillaries;
- FIG. 5C is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 11.4% T acrylamide, 5.7 M urea, and 48.9% (volume:volume) formamide by HPCE (400 V/cm, 4  $\mu$ A) on 10 cm capillaries;

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FIG. 6A is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 14.0% T acrylamide, 5 M urea, and 14% (volume:volume) DMSO;

FIG. 6B is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 14.0% T acrylamide, 5 M urea, and 28% (volume:volume) DMSO;

FIG. 6C is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 14.0% T acrylamide, 5 M urea, and 42% (volume:volume) DMSO;

FIG. 6D is an electropherogram demonstrating the electrophoretic separation of unoxidized heteropolymers differing in length by one base and oxidized homopolymers of polyadenylic acid differing in length by one base, using a substrate composed of 14.0% T acrylamide, 5 M urea, and 56% (volume:volume) DMSO;

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FIG. 7A is an electropherogram demonstrating the electrophoretic separation of unoxidized oligonucleotide analogs 20 to 25 bases in length (SEQ ID NOS 3-8) and homopolymers of polyadenylic acid 19 to 24 bases in length using a substrate composed of 11.4 T acrylamide, 32% (volume:volume) formamide, 7 M urea, and 20% water;

FIG. 7B is an electropherogram demonstrating the electrophoretic separation of unoxidized oligonucleotide analogs 20 to 25 bases in length (SEQ ID NOS 3-8) and homopolymers of polyadenylic acid 19 to 24 bases in length using a substrate composed of 11.4% acrylamide, in 48.9% (volume:volume) formamide, 5.7 M urea;

FIG. 7C is an electropherogram demonstrating the electrophoretic separation of unoxidized oligonucleotide analogs 20 to 25 bases in length (SEQ ID NOS 3-8) and homopolymers of polyadenylic acid 19 to 24 bases in length using a substrate composed of 11.4% acrylamide, in 74% (volume:volume) formamide, 5 M urea, and 0% water; and

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FIG. 8 is an electropherogram demonstrating the electrophoretic separation of unoxidized oligonucleotide analogs 20 to 25 bases in length (SEQ ID NOS:3-7), an oligonucleotide analog (SEQ ID NO:3) with one oxidation, an oligonucleotide analog (SEQ ID NO:3) with two oxidations, and an oligonucleotide analog (SEQ ID NO:3) with twenty-four oxidations, using a substrate composed of 14% T acrylamide, 52% (volume:volume) DMSO, 5 M urea, and 200 mM TBE by HPCE (400 V/cm, 5  $\mu$ A) on a 9 cm capillary.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides a novel substrate and methods of using that substrate to separate  
5 unmodified and modified mononucleotides and unmodified and modified oligonucleotides which may differ by only one base or oxidation.

High performance capillary gel electrophoresis (HPCE) utilizing the novel substrate of the invention holds a unique position in the field of oligonucleotide separation due to its resolution power, ability to determine purity, speed, and automation. Because of the low current generated ( $\mu$ A) from the narrow bore columns (25  $\mu$ m to 200  $\mu$ m, inner diameter), high electric fields (hundreds of volts/cm) without excess Joule heating can be employed, resulting in very rapid, high resolution separations. As an instrumental technique, HPCE is reproducible, amenable to automation, and thus is a powerful alternative tool for antisense analysis.  
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Results obtained by traditional capillary electrophoresis suggest the application of electric fields lower than 200 V/cm with low ionic strength buffer (not higher than 0.1 M Tris-borate-EDTA (TBE)) and low gel concentration in aqueous media for the separation of  
25 oligonucleotides. However, it has been discovered that the use of 0.2 M TBE buffer and an electric field of at least 200 V/cm, and preferably at least 400 V/cm, gives very high resolution in  
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certain gel substrates for the separation of oligonucleotide analogs.

The substrate contains a polymer such as  
5 polyacrylamide, methyl cellulose, polyvinyl alcohol, or derivatives thereof, which may be up to 1% cross-linked but need not be cross-linked at all. It is important that the concentration of polymer in the capillary be 12% or higher to  
10 achieve this kind of resolution and efficiency. No concentration gradient of polymer is required, but linear gradients of, for example, from about 12% to 20%, or more preferably, from about 13% to 18% polymer are useful.

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The polymer is suspended in a solution containing at least 5 M urea. The presence of a high concentration of urea (i.e., at least 5 M, and preferably between 5.7 and 8.3 M) improves  
20 denaturation of the molecules to be separated under the conditions of this method.

The solution also contains at least 32% (volume:volume) organic solvent. Useful organic  
25 solvents are chemically stable liquids at about room temperature, and have a dielectric constant of at least 20. Such solvents include, but are not limited to, methanol, formamide, acetaldehyde, DMSO, ethanol, glycol, acetone, 1-  
30 propanol, 2-propanol, 1, 2-propanediol, 1, 3-propanediol, and glycerol, among others. These solvents, along with their chemical formulae and dielectric constants, are listed below in TABLE 1

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obtained from *The Merck Index* ((10th Ed.) Windholz et al., eds., (1983) Merck & Co., Inc., Rahway, New Jersey).

5

TABLE 1

	<u>Chemical Formula</u>	<u>Solvent</u>	<u>Dielectric Constant</u>	<u>at °C</u>
10	CH <sub>4</sub> O	methanol	33.62	20°
	CH <sub>3</sub> NO	formamide	84.0	20°
	C <sub>2</sub> H <sub>4</sub> O	acetaldehyde	21.0	10°
	C <sub>2</sub> H <sub>6</sub> OS	dimethylsulfoxide	45.0	20°
15	C <sub>2</sub> H <sub>6</sub> O	ethanol	24.30	25°
	C <sub>2</sub> H <sub>6</sub> O <sub>2</sub>	glycol	37.0	25°
	C <sub>3</sub> H <sub>6</sub> O	acetone	20.7	25°
	C <sub>3</sub> H <sub>8</sub> O	1-propanol	20.1	25°
	C <sub>3</sub> H <sub>8</sub> O	2-propanol	18.3	25°
20	C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>	1,2-propanediol	32.0	20°
	C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>	1,3-propanediol	35.0	20°
	C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>	glycerol	42.5	25°

25

In addition, some substrates of the invention include about 14% to 56% DMSO.

30

The substrate consisting of the polymer in the urea/organic solvent solution is placed in a capillary or tube before polymerization in preparation for separation and analysis by HPCE. In the case of acrylamide, polymerization may be achieved by adding ammonium persulfate and a free radical catalyst such as N,N,N',N'-tetramethylene-

35

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diamine (TEMED) to the acrylamide solution. Alternatively, photopolymerization may be used. The substrate solution is then placed into the capillary where it polymerizes. A useful 5 capillary is a microcapillary column (25 to 200  $\mu\text{m}$  inner diameter) made of fused silica, as described in U.S. Patent Nos. 4,865,706 and 5,112,460, herein incorporated by reference. Of course, other initiators and modes of polymerization may 10 be used depending on the type of polymer present in the substrate.

The sample solution containing the molecules to be analyzed is then applied to the substrate in 15 the capillary. The molecules which can be successfully separated on this substrate include unmodified mononucleotides and oligonucleotides and modified mononucleotides and oligonucleotides (or mononucleotide analogs and oligonucleotide 20 analogs) such as oxidized and unoxidized mononucleotides and oligonucleotides, 3'-substituted mononucleotides and oligonucleotides, 5'-substituted mononucleotides and oligonucleotides, 25 3',5'-substituted mononucleotides and oligonucleotides, mononucleotides and oligonucleotide analogs having at least one phosphate group replaced with a chemical group such as alkylphosphonates, phosphorothioates, phosphorodithioates, 30 alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, carbonates, phosphate triesters, acetamide, and

-23-

carboxymethyl esters, among others (see, e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

5       The preparation of these modified and unmodified mononucleotides and oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends in Biotechnol.* 10:152-158). For example, monomeric and oligomeric phosphorothioate analogs can be prepared using methods well known in the 10 field such as methoxyphosphoramidite (see, e.g., Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083) or H-phosphonate (see, e.g., Froehler (1986) *Tetrahedron Lett.* 27:5575-5578) chemistry. The synthetic methods described in Bergot et al. (J. 15 *Chromatog.* (1992) 559:35-42) can also be used.

20       The products of any of these syntheses may include failure sequences as well as the desired oligonucleotide sequence. The failure sequences have at least one less base than the desired oligonucleotide, but the position of the missing base is unknown without subsequent sequencing analysis.

25       In order to separate the failure sequences from the desired oligonucleotides so produced, or in order to distinguish, characterize, and isolate different desired mononucleotides and/or oligonucleotide species from each other, the 30 molecules to be examined are analyzed by HPCE using a capillary electrophoresis apparatus. Such an instrument is well known in the field (see, e.g.,

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Cohen et al. (1988) *Proc. Natl. Acad. Sci.* (USA) 85:9660-9663). The sample is electrophoretically injected into the column by dipping the cathodic end of the capillary into the sample solution and applying a field of 400 V/cm for 1 to 3 sec. The sample is then run through the gel, and the separated analogs detected by UV, infrared, fluorescence, laser-induced fluorescence or other external monitoring methods.

10

As demonstrated by the electropherograms shown in FIGS. 1A and 1B, this method enables the separation of oligonucleotide analogs differing in length by only one base. In addition, this method 15 enables the separation of oligonucleotides differing in their state of oxidation by only one oxidized nonbridging group as demonstrated in FIG. 8.

20

The following examples illustrate the preferred mode of making and practicing the present invention, but are not meant to limit the scope of the invention.

25

#### EXAMPLES

##### 1. HPCE Apparatus

30 The high performance capillary electrophoresis apparatus with UV detection and the preparation of substrate-filled capillary for the separation of DNA molecules are essentially the same as described in Cohen et al. (*Proc. Natl.*

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Acad. Sci. (USA) (1988) 85:9660-9663) and Heiger et al. (J. Chromatogr. (1990) 516:33-48), herein incorporated by reference. A 30 kV, 500  $\mu$ A direct current high voltage power supply (Model ER/DM; 5 Glassman, Whitehouse Station, NJ) is used to generate the potential across the capillary.

## 2. Preparation of Substrate-Filled Capillaries

10 Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ) with inner diameter of 75  $\mu$ m, outer diameter of 375  $\mu$ m, effective length of 20 cm, and total length of 30 cm, is treated with (methylacryloxypropyl)trimethoxysilane  
15 (Petrarch Systems, Bristol, PA) and then filled with degassed 13 to 18% T polymerizing acrylamide in aqueous or organic solution in 0.2 M TBE buffer (0.2 M Tris borate, 4 mM EDTA), pH 8.3, with 7 M to 8.3 M urea. Alternatively, capillaries are  
20 filled with a degassed 0.2 M TBE buffer solution of 11.4%, 13%, or 18% T acrylamide, in 32% to 74% (volume:volume) formamide, 5.7 M urea; or 14% T acrylamide in 14% to 56% (volume:volume) DMSO, 5 M urea; or 12.6% to 14% T acrylamide in 5 to 7.4 M urea and 40.5% (volume:volume) DMSO.  
25 Polymerization is achieved by adding ammonium persulfate solution and TEMED. To remove impurities from the polyacrylamide, the capillary column is pre-electrolyzed at 6 kV for 30 to 60 minutes. During electrophoresis, the capillary is maintained at room temperature. Ultra-pure Trizma base, urea, acrylamide, and EDTA are purchased  
30 from Schwartz/Mann Biotech (Cleveland, OH). TEMED

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and ammonium persulfate are purchased from Bio-Rad (Richmond, CA).

### 3. Preparation of Oligonucleotides

5

The oligonucleotide phosphorothioate 25mer 5'-CGTATAGCCTGATGTCATAGCCGAT-3' (SEQ ID NO:1), 24-  
mer 5'-GACTCGAGGTCTGCTAACCTAGAT-3' (SEQ ID NO:2),  
25mer 5'-CTCTCGCACCCATCTCTCCTTCT-3' (SEQ ID  
10 NO:3), 24mer 5'-TCTCGCACCCATCTCTCCTTCT-3' (SEQ  
ID NO:4), 23mer 5'-CTCGCACCCATCTCTCCTTCT-3' (SEQ  
ID NO:5), 22mer 5'-TCGCACCCATCTCTCCTTCT-3' (SEQ  
ID NO:6), 21mer 5'-CGC-ACCCATCTCTCCTTCT-3' (SEQ  
ID NO:7), 20mer 5'-CGCACC-CATCTCTCCTTCT-3' (SEQ  
15 ID NO:8), poly d[T] analogs ranging in length from  
19 to 24 bases, and the failure sequences from the  
syntheses of various oligomers having a length of  
up to 150 bases (base sequences unknown) are  
synthesized using the procedure of Beaucage et al.  
20 (U.S. Patent No. 5,003,097), herein incorporated  
by reference. Briefly, oligodeoxyribonucleotides  
are synthesized on an automated synthesizer (Model  
8700, Milligen/Bioscience, Bedford, MA). Both  
normal phosphodiester oligodeoxyribonucleotides  
25 and their phosphorothioate analogs are assembled  
using H-phosphonate chemistry (Andrus et al.  
(1988) *Tetrahedron Lett.* 29:61; Gregg et al. (1987)  
*Tetrahedron Lett.* 27:4051). Synthesis is carried out  
on a 10-μmol scale, and after the chain elongation  
30 cycles the controlled pore glass support-bound  
oligonucleoside. H-phosphonate is treated either  
with 0.2 M sulfur in carbon disulfide:pyridine:

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triethylamine (12:12:1, volume:volume) to generate phosphorothioate internucleotide linkages. Deprotection of oligodeoxyribonucleotide is carried out with concentrated ammonia at 55°C for 5 hours. Deprotected oligodeoxyribonucleotides are then resuspended in distilled water.

#### 4. Separation of Oligonucleotides

10 Samples are electrophoretically injected into the column by dipping the cathodic end of the capillary into the sample solution and applying a voltage of 400 V/cm for 2 seconds. Separation is achieved at a typical applied field of from 400 to 15 800 V/cm. Each column is used for multiple injections. Periodically, a short section of the capillary at the injection end is trimmed.

20 The failure sequence sample (containing oligonucleotides varying in length from 1 to 50 bases, from 1 to 75 bases, and from 1 to 150 bases is suspended in water with final concentration 500 ng/ $\mu$ l. Each of these samples is separated on a 25 capillary containing 15% T acrylamide. The column is developed with 60% (volume:volume) formamide, 0.2 M TBE buffer, 8.3 M urea, pH 8.3. Electrophoresis is conducted under an applied electric field of 400 volts/cm and a current of 5  $\mu$ A over a 20 cm migration distance. The results 30 from the 1 to 50 and 1 to 75 base samples are shown in FIGS. 1A and 1B. When migration time is examined with respect to fragment length, a linear relationship ( $r^2=0.9999$ ) is observed (FIG. 2).

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This linear behavior of the phosphorothioate analogs is indicative of the lack of peak compression, and of migration according to molecular weight or size, each being important  
5 elements of successful oligonucleotide separation.

A sample containing a mixture of the 24mer (SEQ ID NO:2) and the 25mer (SEQ ID NO:1) phosphorothioate analogs (having different sequences but the same length) is suspended in water to final concentration 400 ng/ml. The sample is run on a capillary containing 13% T, 0% C, 7 M urea, 0.2 M TBE, pH 8.3. (The term "c" refers to a fraction: the amount of crosslinked polymer over the total monomer and cross-linked monomer). Electrophoresis is conducted under an electric field of 400 volts/cm and a current of 12 µA over a 20 cm migration distance. The results of this separation are shown in FIG. 3. The time window between elution of the 24mer (SEQ ID NO:2) and elution of the 25mer (SEQ ID NO:1) is large enough to accommodate an additional peak. This peak is presumed to be a failure sequence of the synthesized 25mer and is therefore a 24mer since this peak is migrating directly after the 25mer under denaturing conditions. Thus, the two 24mers may be separated due to the difference in their base sequences.  
20  
25

30 In other tests, heteropolymers of unoxidized phosphorothioate oligonucleotide 20-25mer analogs (SEQ ID NOS: 8, 7, 6, 5, 4, and 3), oxidized homopolymers of polyadenylic acid 19-24 bases in

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length, and mixtures thereof, are resolved on 10 cm 12.6% T acrylamide capillaries containing 40.5% (volume:volume) DMSO and 7.4 M urea at 800 V/cm, 3  $\mu$ A (FIGS. 4A-4C), on 10 cm 14% T acrylamide capillaries containing 14% to 56% DMSO and 5.0 urea at 800 V/cm, or, on 10 cm 11.4% T acrylamide capillaries containing 74% (volume:volume) formamide and 5.7 M urea at 400 V/cm, 4  $\mu$ A (FIG. 7C).

10

The results show that a substrate containing 40.5% (volume:volume) DMSO and 16.2% water (FIG. 4C) enables the separation of a mixture of completely unoxidized oligonucleotides (e.g., phosphorothioates) differing in length by only 1 base and completely oxidized oligonucleotides (e.g., phosphodiesters) differing in length by only one base, in contrast to a substrate wherein DMSO is substituted for formamide (FIG. 5C).

20

Substrates containing 28% or lower concentration of DMSO are comparable in the separation abilities to that of 48.9% or lower formamide-containing substrates (FIG. 5C). In addition, a substrate containing 74% formamide, 5 to 5.7 M urea, and 0% water, is found to separate a mixture of oxidized and unoxidized oligonucleotide analogs differing in length by only one base (FIG. 7C). These results are comparable to the results shown in FIGS. 6C and 6D wherein the substrate contains 42% and 56% (volume:volume), respectively, DMSO.

25

30

Thus, the results demonstrate that by increasing organic solvent concentration, improved

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separation of oligonucleotides differing in length by only one base and by oxidation state can be obtained. Furthermore, substrates containing 14% T acrylamide, 52% (volume:volume) DMSO, 5 M urea, 5 and 200 mM TBE can separate unoxidized phosphorothioate heteropolymers 20 to 25 bases in length (SEQ ID NOS:3-8) from each other and from a heteropolymeric analog 25 bases in length (SEQ ID NO:3) having one or two oxidations (see FIG. 8, 10 peaks A and B, respectively; peak C is a 25mer with 24 oxidations).

##### 5. Detection Method

15 Oligonucleotides are monitored by UV detection at wavelength 270 nm using a Spectra-100 spectrophotometer (Spectra Physics, San Jose, CA). The data are stored on an Ace IBM compatible PC computer via an analog to digital (A/D) converter 20 (Model 970, Nelson Analytical, Cupertino, CA).

Those skilled in the art will recognize, or 25 be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

-31-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANTS: Cohen, Aharon S.  
Bourque, Andre  
Vilenchik, Maria

10

(ii) TITLE OF INVENTION: Method for Analyzing  
Oligonucleotide Analogs

15 (iii) NUMBER OF SEQUENCES: 8

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lappin & Kusmer  
(B) STREET: 200 State Street  
(C) CITY: Boston  
(D) STATE: Massachusetts  
20 (E) COUNTRY: U.S.A.  
(F) ZIP: 02109

20

(v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kerner, Ann-Louise  
(B) REGISTRATION NUMBER: 33,523  
(C) REFERENCE/DOCKET NUMBER: HYZ-012PCT

40

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617/330-1300  
(B) TELEFAX: 617/330-1311

(2) INFORMATION FOR SEQ ID NO:1:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

-32-

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGTATAGCCT GATGTCATAG CCGAT

25

10

(2) INFORMATION FOR SEQ ID NO:2:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GACTCGAGGT CTGCTAACCT AGAT

24

30

(2) INFORMATION FOR SEQ ID NO:3:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

45

CTCTCGCACCATCTCTCTCTTCT

25

50

-33-

(2) INFORMATION FOR SEQ ID NO:4:

- 5                   (i) SEQUENCE CHARACTERISTICS:  
                      (A) LENGTH: 24 base pairs  
                      (B) TYPE: nucleic acid  
                      (C) STRANDEDNESS: single  
                      (D) TOPOLOGY: linear

10                  (ii) MOLECULE TYPE: cDNA

                     (iii) HYPOTHETICAL: NO

15                  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

20                  TCTCGCACCC ATCTCTCTCC TTCT

24

25                  (2) INFORMATION FOR SEQ ID NO:5:

- 20                   (i) SEQUENCE CHARACTERISTICS:  
                      (A) LENGTH: 23 base pairs  
                      (B) TYPE: nucleic acid  
                      (C) STRANDEDNESS: single  
                      (D) TOPOLOGY: linear

25                  (ii) MOLECULE TYPE: cDNA

30                  (iii) HYPOTHETICAL: NO

                     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35                  CTCGCACCCA TCTCTCTCCT TCT

23

40                  (2) INFORMATION FOR SEQ ID NO:6:

- 40                   (i) SEQUENCE CHARACTERISTICS:  
                      (A) LENGTH: 22 base pairs  
                      (B) TYPE: nucleic acid  
                      (C) STRANDEDNESS: single  
                      (D) TOPOLOGY: linear

45                  (ii) MOLECULE TYPE: cDNA

                     (iii) HYPOTHETICAL: NO

-34-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCGCACCCAT CTCTCTCCTT CT

22

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

20

CGCACCCATC TCTCTCCTTC T

21

25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

40

GCACCCATCT CTCTCCTTCT

20

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What is claimed is:

1. A method of separating unmodified and/or modified mononucleotides and/or oligonucleotides,  
5 the method comprising the steps of:

(a) providing a substrate in a high performance capillary, the substrate comprising at least 12% (weight:volume)  
10 polymer, at least 5 M urea and at least 32% (volume:volume) organic solvent, the organic solvent being a chemically stable liquid at room temperature and having a dielectric constant of at least 20;

15 (b) contacting the substrate with the mononucleotides and/or oligonucleotides to be separated;

20 (c) applying an electric field greater than 200 volts/cm across the gel in the capillary; and

25 (d) detecting the separated mononucleotides and/or oligonucleotides.

2. The method of claim 1 wherein the providing step (a) comprises providing a substrate which includes from 0 to 16.2% (volume:volume) water.

30

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3. The method of claim 1 wherein the providing step (a) comprises providing a substrate which comprises a polymer selected from the group consisting of polyacrylamide, methylcellulose, and polyvinyl alcohol.  
5
4. The method of claim 3 wherein the providing step (a) comprises providing a substrate including polyacrylamide.  
10
5. The method of claim 4 wherein the providing step (a) comprises providing a substrate containing from about 12% to 20% T acrylamide.  
15
6. The method of claim 5 wherein the providing step (a) comprises providing a substrate containing about 18% T acrylamide.  
20
7. The method of claim 4 wherein the providing step (a) comprises providing a substrate containing a linear gradient of from about 12% to 18% T acrylamide.  
25
8. The method of claim 1 wherein the providing step (a) comprises providing a non-cross-linked substrate.  
30
9. The method of claim 1 wherein the providing step comprises providing a substrate which is up to about 1% cross-linked.

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10. The method of claim 1 wherein the providing step (a) comprises providing a substrate containing 5 M to 8.3 M urea.

5       11. The method of claim 1 wherein the providing step (a) comprises providing a substrate containing an organic solvent selected from the group consisting of methanol, formamide, acetaldehyde, ethanol, dimethylsulfoxide,  
10      glycol, acetone, 1-propanol, 2-propanol, glycerol, 1, 2-propanediol, and 1, 3-propanediol.

15      12. The method of claim 11 wherein the providing step (a) comprises providing a substrate containing formamide.

20      13. The method of claim 12 wherein the providing step (a) comprises providing a substrate containing about 32% to 74% (volume:volume) formamide.

25      14. The method of claim 11 wherein the providing step (a) comprises providing a substrate containing dimethylsulfoxide.

15. The method of claim 14 wherein the providing step (a) comprises providing a substrate containing about 32% to 52% (volume:volume) dimethylsulfoxide.

30

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5           16. The method of claim 1 wherein the contacting step (b) comprises contacting the substrate with a modified mononucleotide selected from the group consisting of 3'-substituted mononucleotide analogs, 5'-substituted mononucleotide analogs, and 3', 5'- substituted mononucleotide analogs.

10          17. The method of claim 16 wherein the contacting step (b) comprises contacting the substrate with a mononucleotide analog having a 5'-linked chemical structure selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, and phosphate esters, carbamates, carbonates, phosphate triesters, acetamide, and carboxymethyl esters.

15          18. The method of claim 1 wherein the contacting step (b) comprises contacting the substrate with a modified oligonucleotide having at least one synthetic internucleotide linkage.

20          19. The method of claim 18 wherein the contacting step (b) comprises contacting the substrate with an oligonucleotide analog having at least one synthetic internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, carbonates, phosphate triesters, acetamide, and carboxymethyl esters.

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20. The method of claim 19 wherein the contacting step (b) comprises contacting the substrate with a modified mononucleotide having a 5'-phosphorothioate substitution and a modified oligonucleotide having at least one phosphorothioate internucleotide linkage.
- 5
21. The method of claim 1 wherein the contacting step (b) comprises contacting the substrate with a modified oligonucleotide having at least one unoxidized substitution at a nonbridging oxygen.
- 10
22. The method of claim 1 wherein the contacting step (b) comprises contacting the substrate with mononucleotides and oligonucleotides having from 1 to 150 bases.
- 15
23. The method of claim 22 wherein the contacting step (b) comprises contacting the substrate with mononucleotides and oligonucleotides having from 1 to 50 bases.
- 20
24. The method of claim 1 wherein the applying step (c) comprises applying an electric field of about 400 volts/cm across the substrate.
- 25
25. The method of claim 1 wherein the applying step (c) comprises applying an electric field of about 800 volts/cm across the substrate.
- 30

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26. A substrate useful for separating unmodified and/or modified mononucleotides and oligonucleotides, the substrate comprising at least 12% (weight:volume) polymer in at least 5 M urea and at least 32% (volume:volume) organic solvent, the organic solvent being a chemically stable liquid at room temperature and having a dielectric constant of at least 20.
- 5
- 10 27. The substrate of claim 26 comprising from 0 to 16.2% (volume:volume) water.
- 15 28. The substrate of claim 26 wherein the substrate comprises a polymer selected from the group consisting of polyacrylamide, methylcellulose, and polyvinyl alcohol.
- 20 29. The substrate of claim 28 comprising polyacrylamide.
30. The substrate of claim 29 comprising from about 12 to 20% T acrylamide.
- 25 31. The substrate of claim 29 comprising a linear gradient of from about 13 to 18% T acrylamide.
32. The substrate of claim 26 comprising a non-cross-linked substrate.
- 30 33. The substrate of claim 32 which is up to 1% cross-linked.

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34. The substrate of claim 26 comprising from 5 M to 8.3 M urea.

5       35. The substrate of claim 26 wherein the organic solvent is selected from the group consisting of methanol, formamide, acetaldehyde, ethanol, dimethylsulfoxide, glycol, acetone, 1-propanol, 2-propanol, glycerol, 1, 2-propanediol, and 1, 3-propanediol.

10

36. The substrate of claim 35 wherein the organic solvent is formamide.

15

37. The substrate of claim 36 comprising about 32% to 74% (volume:volume) formamide.

38. The substrate of claim 35 wherein the organic solvent is dimethylsulfoxide.

20

39. The substrate of claim 38 wherein the substrate comprises about 32% to 52% (volume:volume) dimethylsulfoxide.

25

40. A substrate useful for separating unmodified and/or modified mononucleotides and oligonucleotides, the substrate comprising about 14% to 56% (weight:volume) dimethylsulfoxide in at least 5 M urea and at least 32% (volume:volume) organic solvent, the organic solvent being a chemically stable liquid at room temperature and having a dielectric constant of at least 20.

30

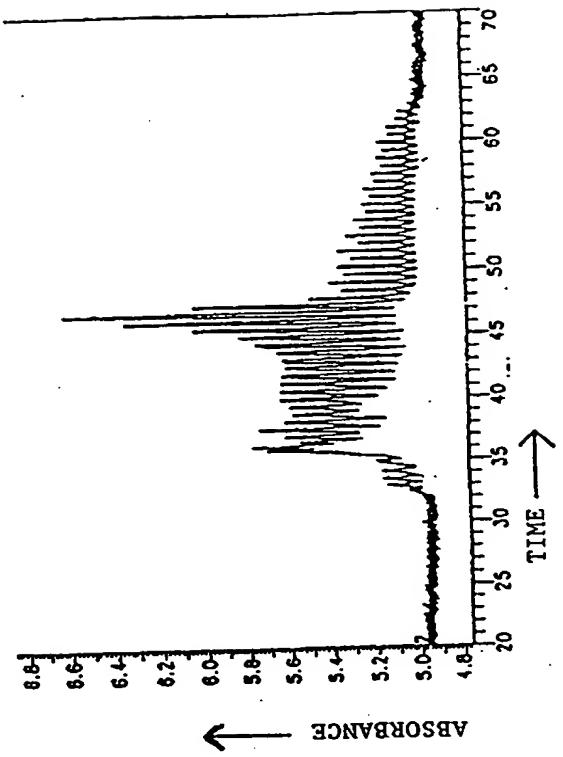


FIG. 1A

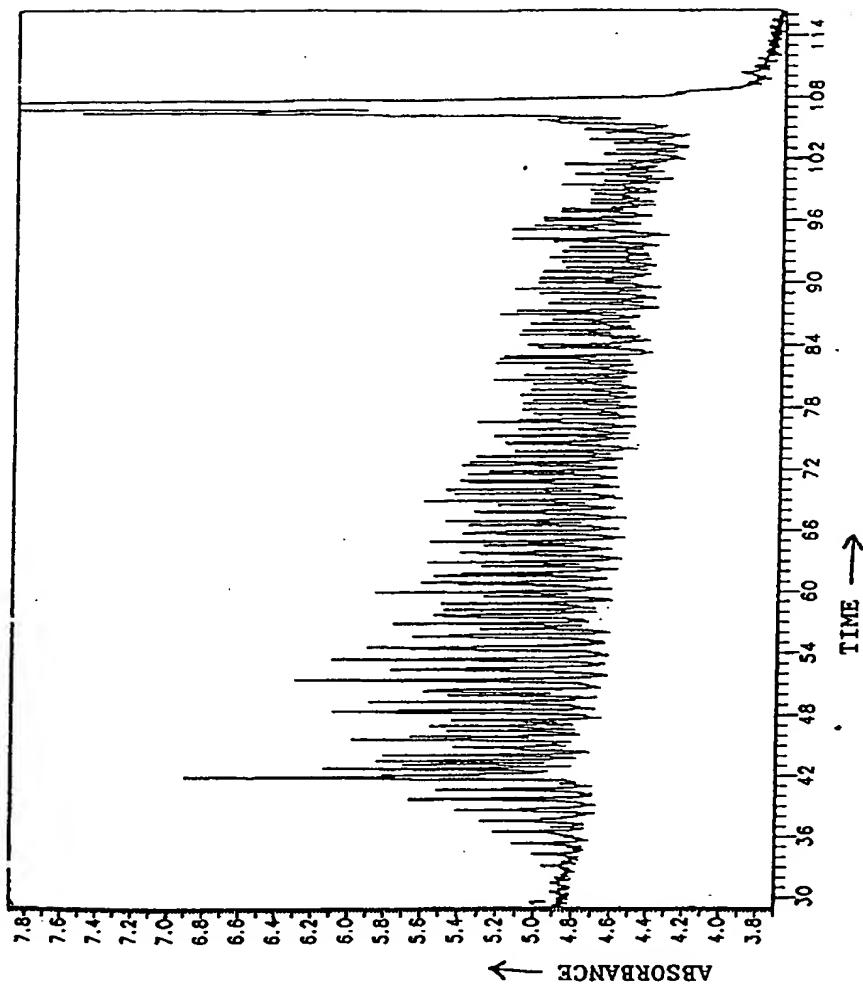


FIG. 1B

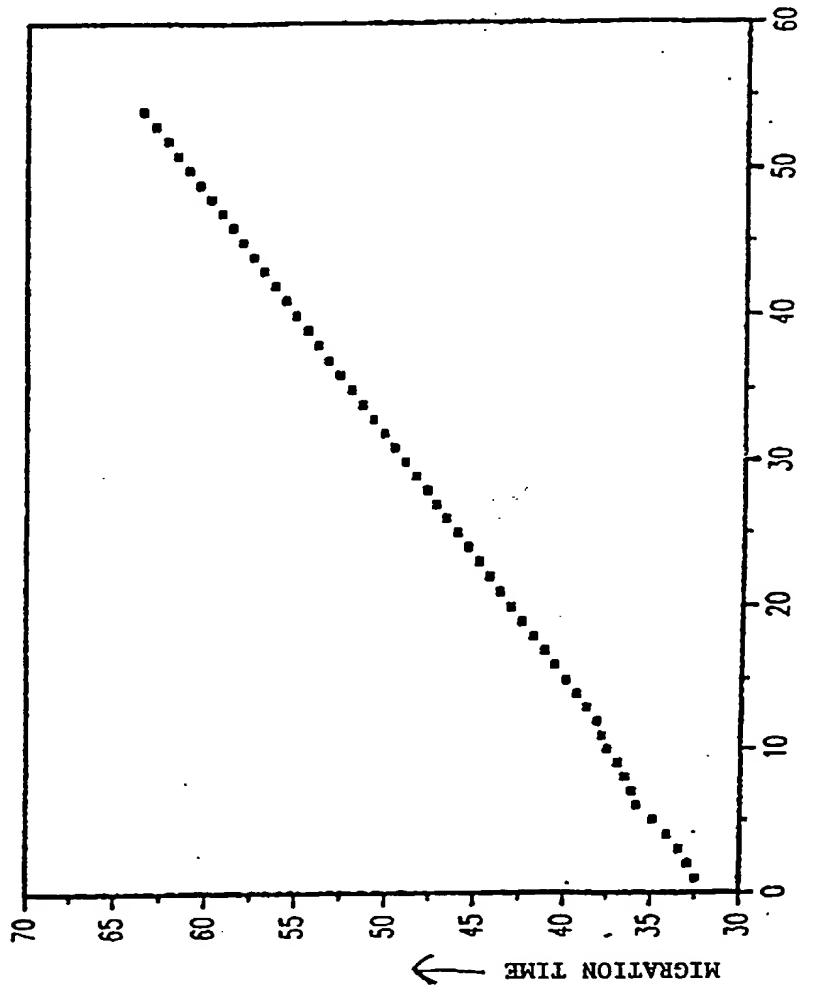


FIG. 2

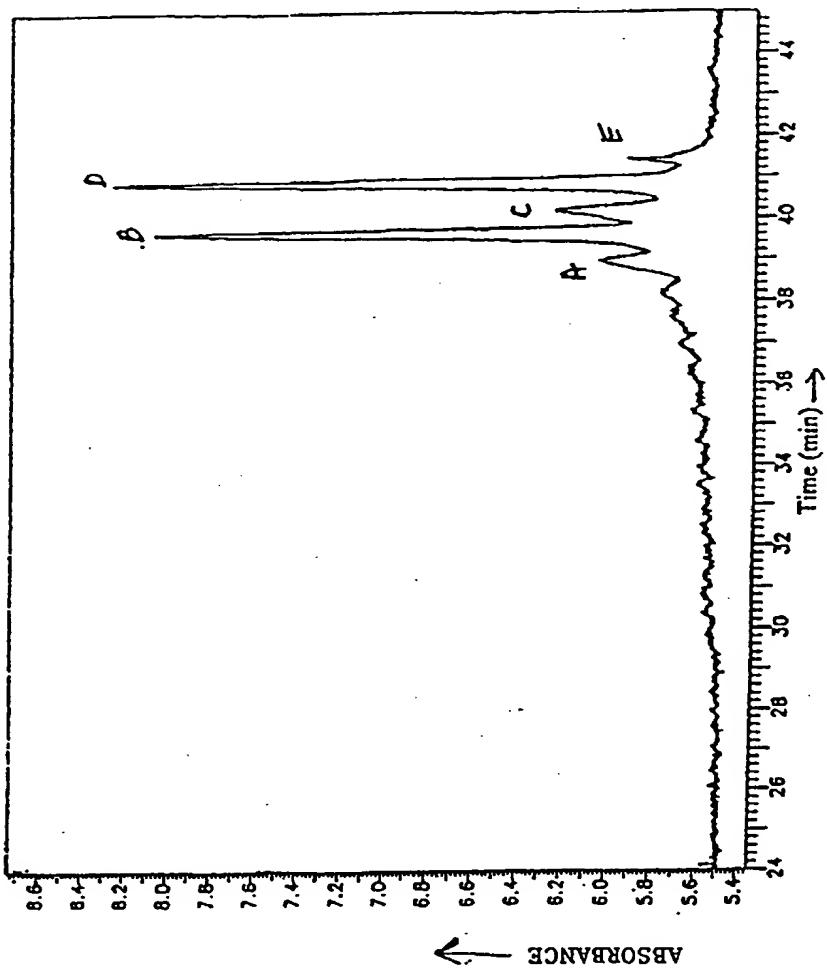


FIG. 3

FIG. 4A

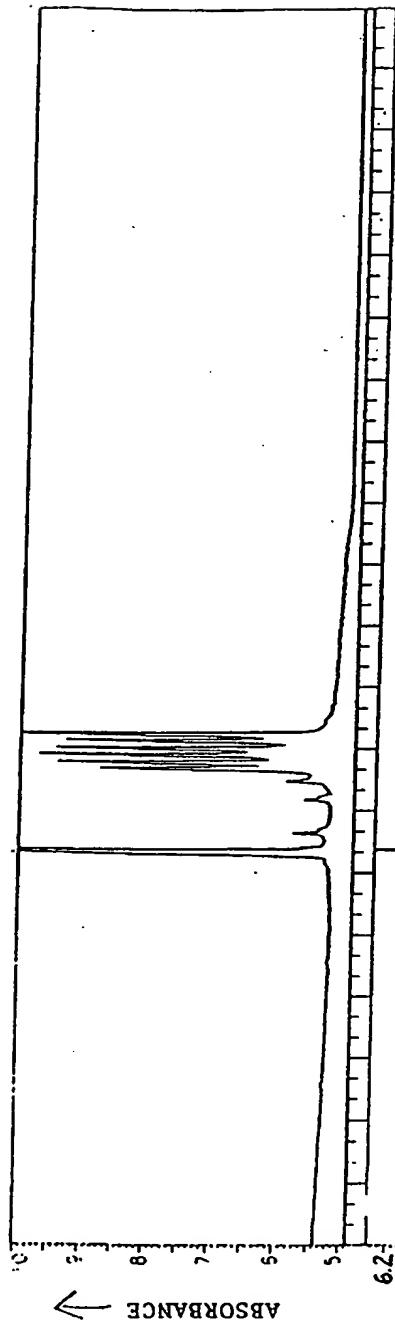


FIG. 4B

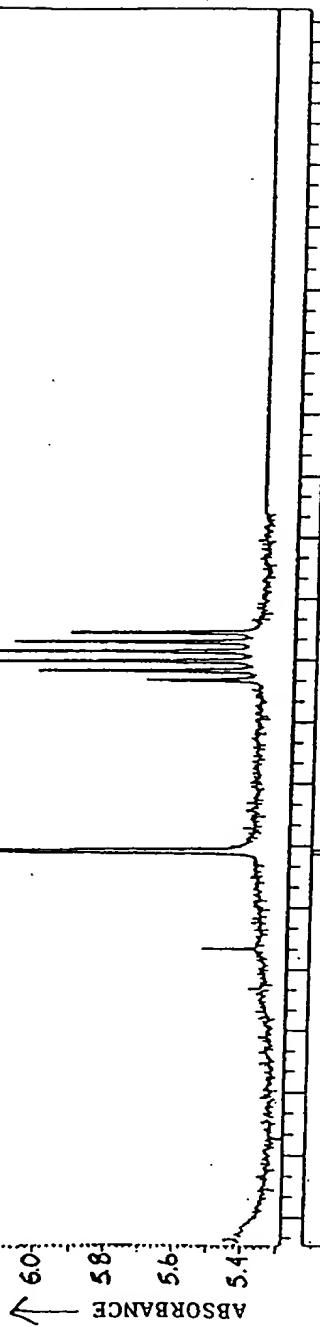


FIG. 4C

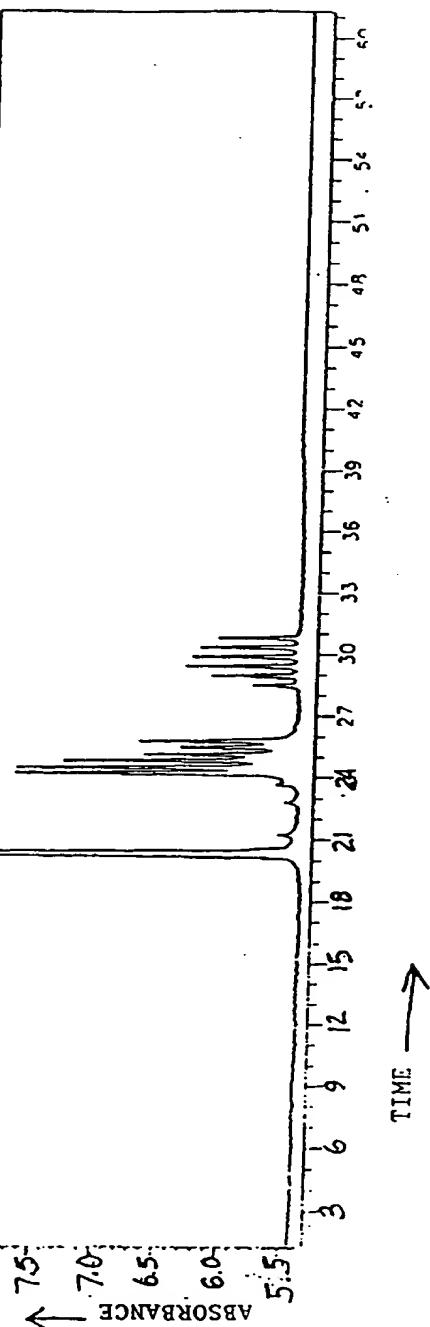


FIG. 5A

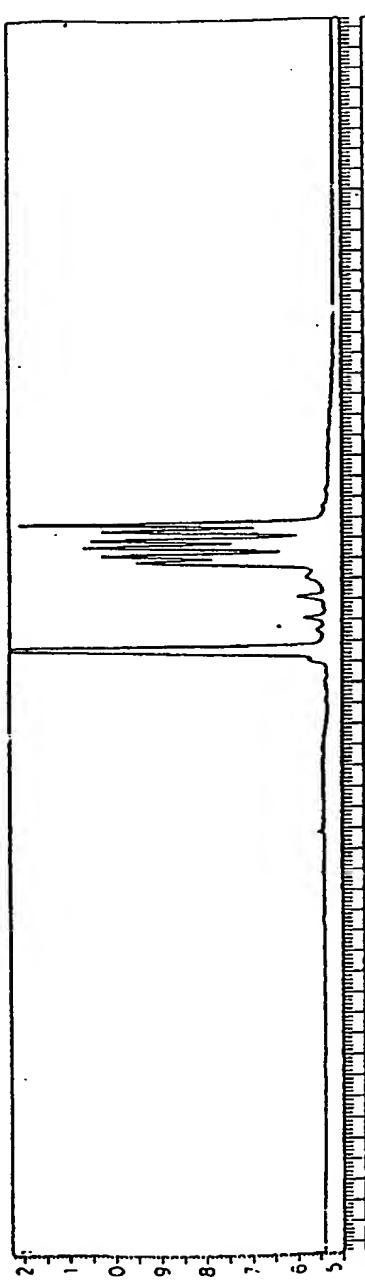


FIG. 5B

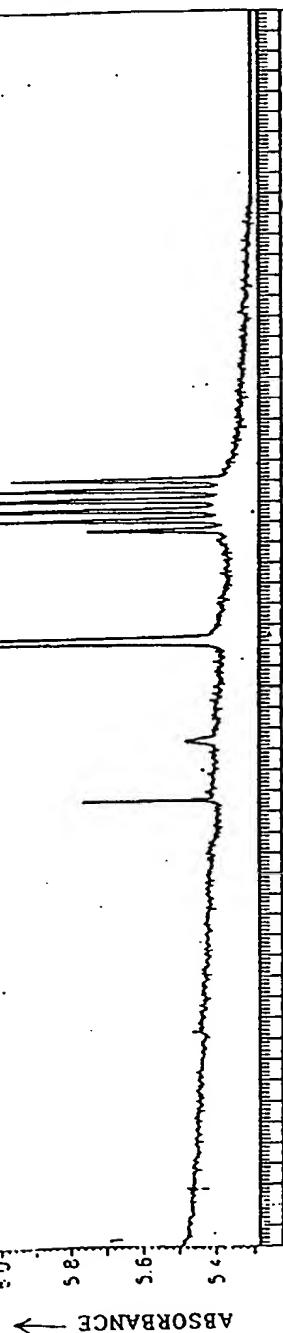


FIG. 5C

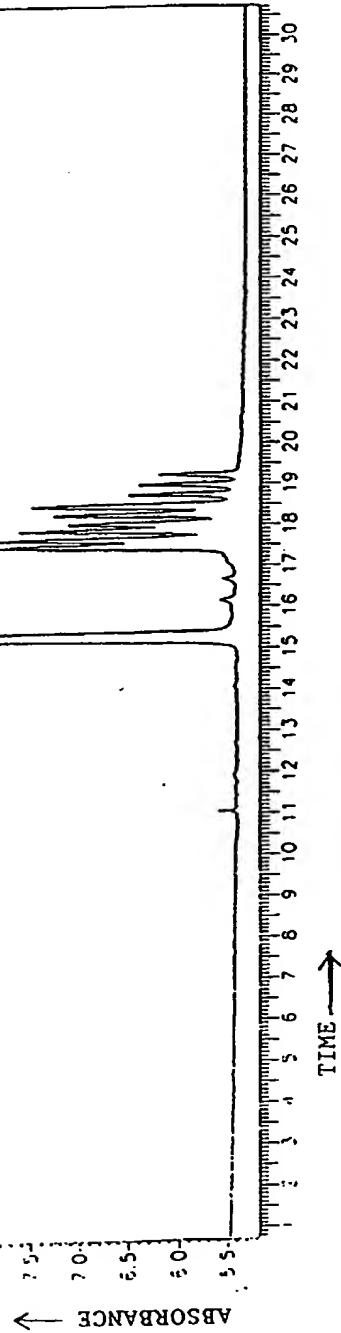


FIG. 6 A

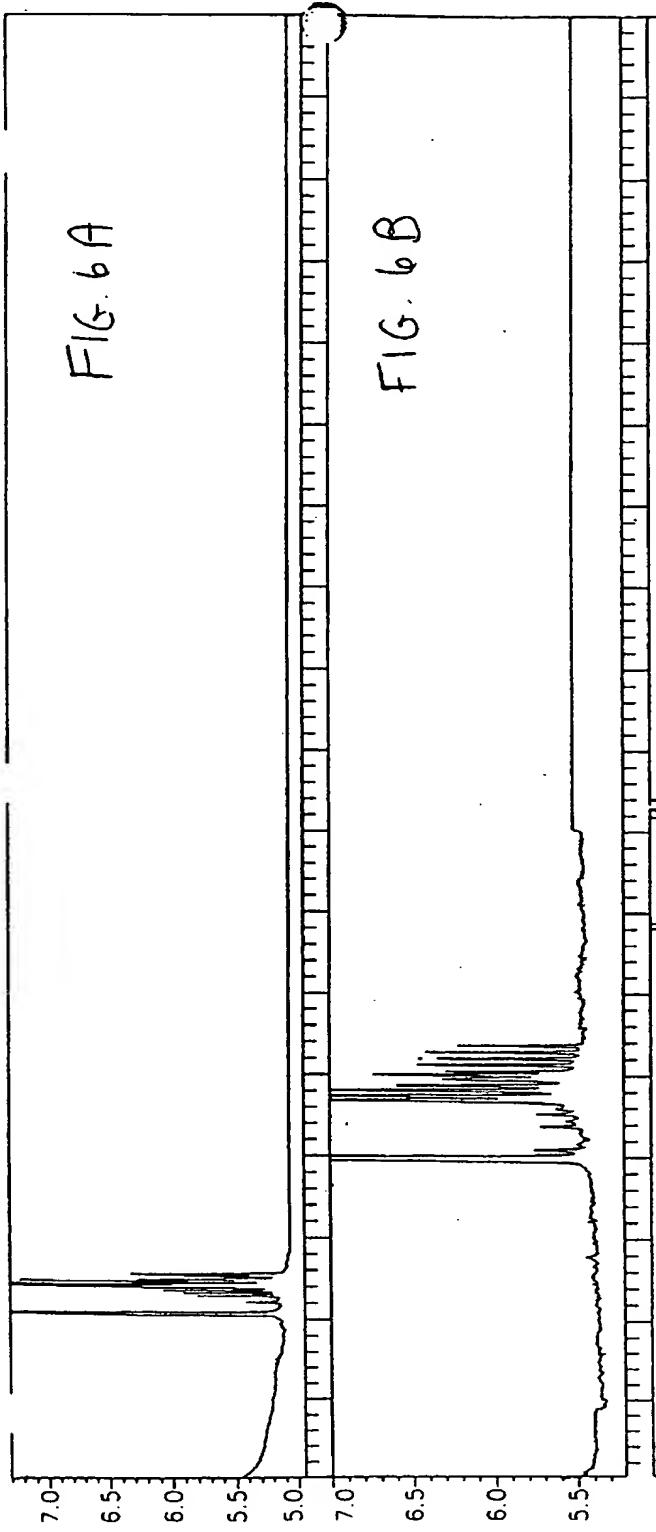


FIG. 6 B

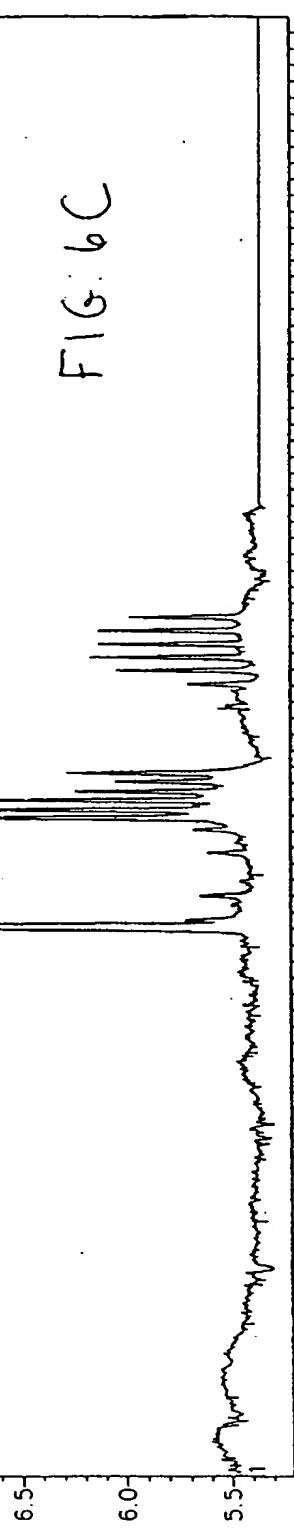


FIG. 6 C

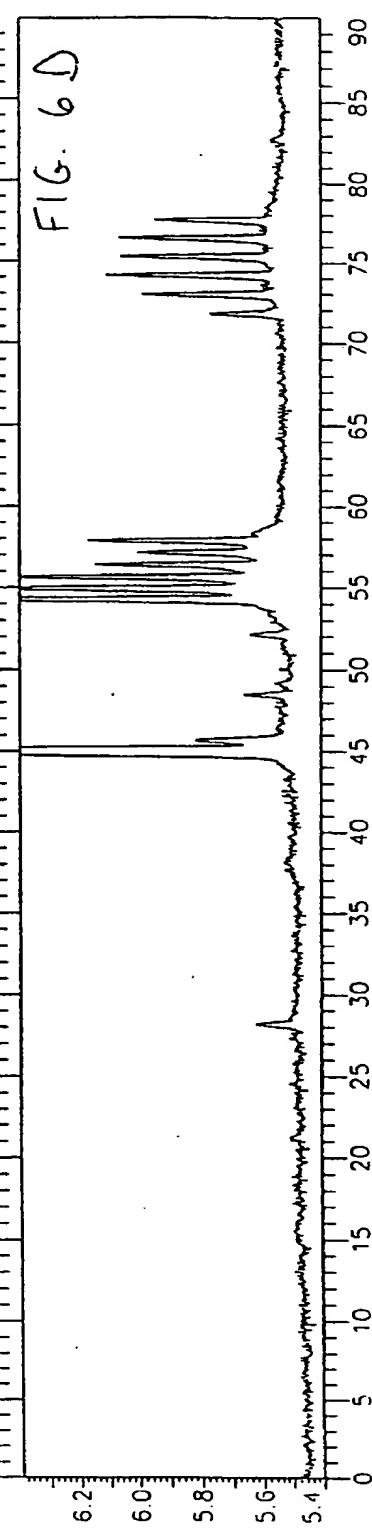


FIG. 6 D

FIG. 7 A

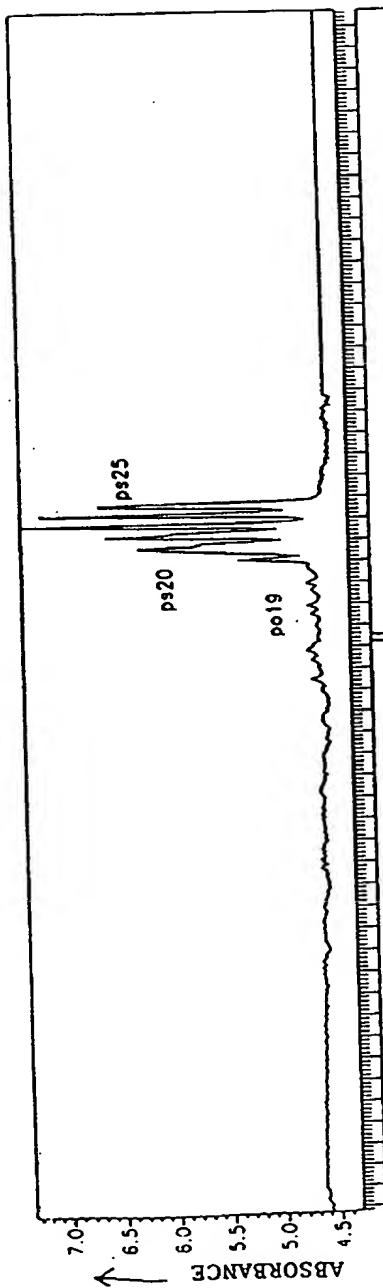


FIG. 7 B

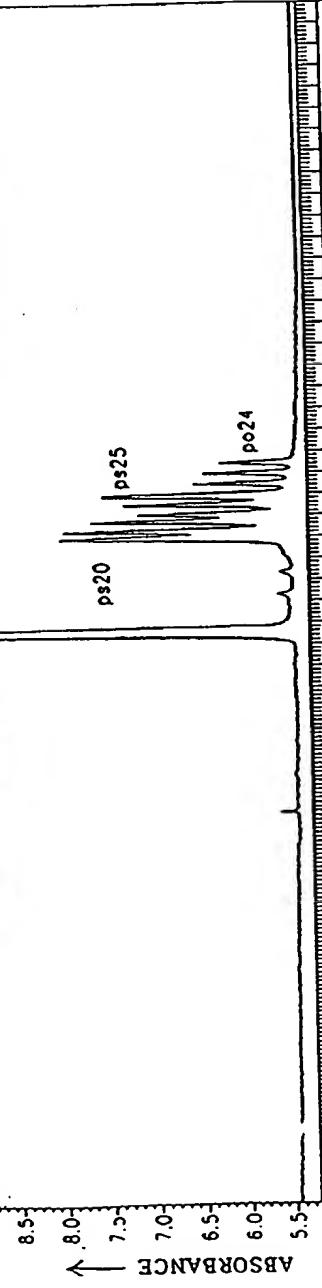
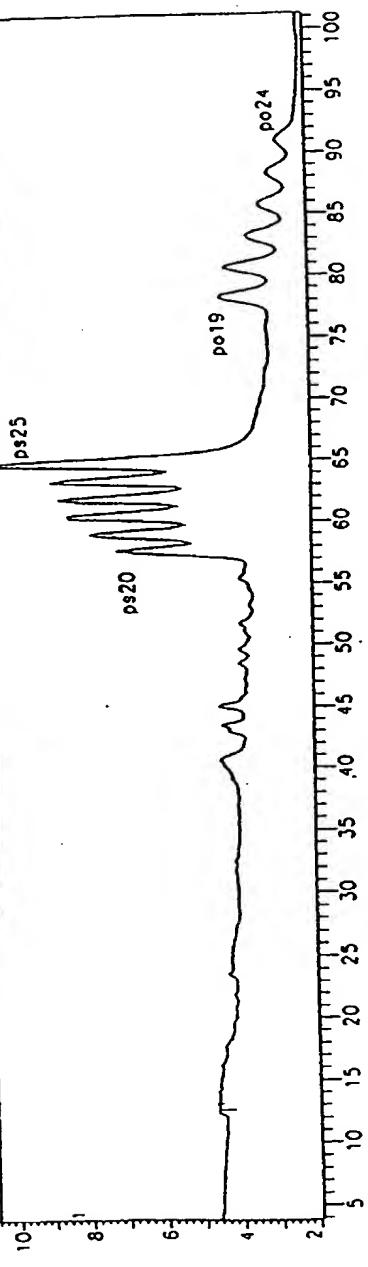


FIG. 7 C



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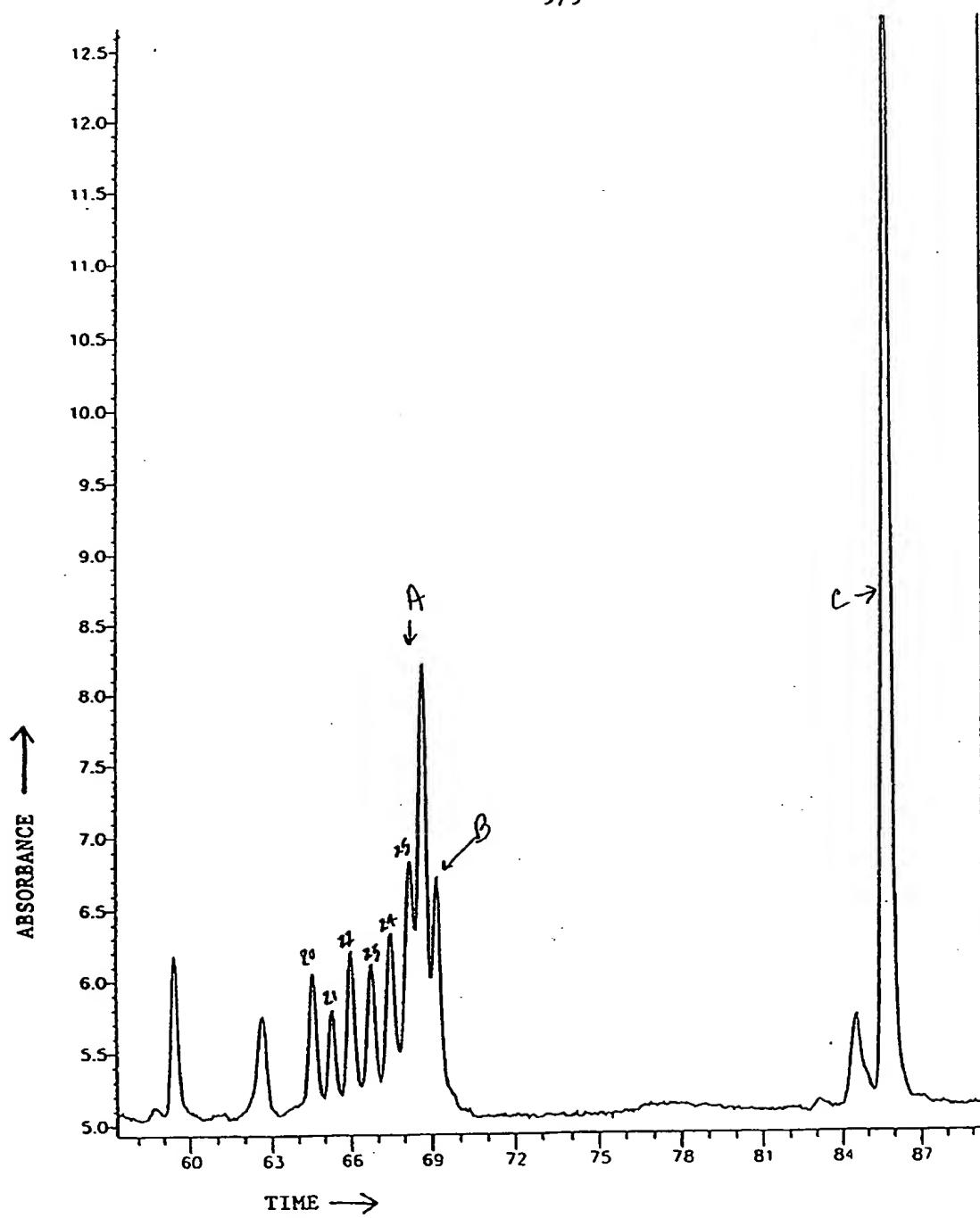


FIG. 8

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/00163

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07H1/06 C07H21/00 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07H GO1N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 497 448 (BECKMANN INSTRUMENTS INC) 5 August 1992 see the whole document ---	1-13, 26-37
P,X	WO,A,94 14824 (HYBRIDON INC.) 7 July 1994 see the whole document ---	1-13, 16-37
X	TRENDS IN ANALYTICAL CHEMISTRY, vol. 12,no. 5, 1993 AMSTERDAM NL, pages 195-202, COHEN A.S. ET AL 'Capillary gel electrophoresis of biopolymers' see page 200, column 2 ---	1,16,26

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

3 May 1995

Date of mailing of the international search report

19.05.95

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Day, G

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/00163

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF CHROMATOGRAPHY B, vol. 662, no. 2, February 1994 AMSTERDAM NL, pages 343-349, BOURQUE A.J. AND COHEN A.S. 'Quantitative analysis of phosphorothioate oligonucleotides in biological fluids using direct injection fast anion-exchange chromatography and capillary gel electrophoresis' see page 344, column 2 -----	1,16,26

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

**PCT/US 95/00163**

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-497448	05-08-92	JP-A-	5093710	16-04-93
WO-A-9414824	07-07-94	AU-B-	6441594	19-07-94